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13. ABSTRACT *(Maximum 200 Words)*

These studies are designed to determine whether ethanol antagonizes the ability of the skeleton to adapt to increased mechanical usage. Ethanol reversibly alters the biophysical properties of cell membranes. The overall hypothesis to be tested in adult rats is that these membrane changes disrupt essential cell signaling pathways for one or more cytokines, growth factors and polypeptide hormones that regulate bone modeling and remodeling. This report summarizes our progress from the award date (November 1998) through August 31, 1999. During Year 1 of the award we have made progress in completing Tasks 1-5, and Task 8. These tasks include: determination of the dose response and time course effects of administered ethanol (Tasks 1 and 2) on blood alcohol levels, serum chemistry and bone metabolism; determination of which induces a more detrimental skeletal response: peak blood concentration of ethanol or chronic elevation of blood alcohol (Task 3); evaluation of the long-term skeletal effects of ethanol on bone metabolism and strength (Task 4); determination of the effects of ethanol on the skeletal adaptation resistance exercise training (Task 5); determination of the effects of prior consumption of ethanol or PTH-induced increases in mRNA levels for bone matrix proteins (Task 8).

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Russell T. Tum Sept 27, 1999

PI - Signature Date

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(5) INTRODUCTION

Chronic alcohol abuse is an important risk factor for osteoporosis. The ultimate goal of this research is to identify the cellular and molecular mechanisms responsible for mediating ethanol's dose- and time-dependent actions on bone turnover, mass, architecture, and strength. It is well established that ethanol reversibly alters the biophysical properties of cell membranes and in doing so disturbs normal membrane function. The proposed studies in young adult rats will test our working hypothesis that these membrane changes disrupt essential cell signaling pathways for one or more bone cells "coupling" factors and/or polypeptide hormones that regulate bone modeling and remodeling. These changes are postulated to lead to the bone loss associated with chronic alcohol abuse. If our hypothesis is correct, then ethanol antagonizes the ability of the skeleton to respond to weight bearing because the signal transduction pathways for mechanical signals require peptide signaling molecules as intermediates. This latter effect of ethanol to reduce the ability of the skeleton to adapt to increased mechanical stress would be especially detrimental during rigorous military training.

(6) BODY

Introduction

We have completed studies which are relevant to Tasks 1-4, and Task 8. Additionally, we have made progress in completing Task 5.

We will discuss the progress for each task separately. Table 1 lists experiment number, title, and task(s).

Task 1

The goal of this Task was to determine the dose response effects of administered ethanol on serum chemistry and gene expression in bone. Four studies (Experiments 1-4) have been completed (see Table 1).

The dose response effects of administered ethanol on serum ethanol concentration one hour later are shown in Table 2. The lower doses of ethanol (0 to .3 mg/kg) resulted in blood alcohol concentration below the detection limit. Further increases in the dose led to a rapid rise in blood alcohol. Doses greater than 1.2 mg/kg led to blood alcohol levels (>0.01%) which would be considered to lead to functional impairment. Blood alcohol was below the detection limit at all doses when assayed at 6 hours.

Ethanol did not induce the expression of either c-*fos* or c-*jun* (negative data not shown). In contrast, 1 hour treatment with either estrogen (1) or PTH (unpublished data) induces mRNAs for these two immediate response genes.

Representative phospor images of Northern blot and RNase protection assays are shown in Figure 1. Ethanol resulted in dose dependent increases in mRNA levels for osteocalcin, type 1 collagen and osteonectin at the proximal tibial metaphysis. The mRNA levels for all three bone

matrix proteins were significantly elevated at the 1.2 g/kg dose. A similar increase was observed in vertebrae (Figure 3). The dose response effects of ethanol on mRNA levels for representative cytokines and growth factors are shown in Figure 4. Ethanol resulted in a dose dependent increase in the RNA levels for TNF-α (significantly elevated at the two highest doses) and a dose dependent decrease in the mRNA for IGF-I (significantly reduced at the highest dose).

Task 2

The goal of this Task is to establish the time course effects of ethanol on the gene expression of bone matrix proteins and signaling peptides. Three studies have been performed (Table 1).

The time course effects of ethanol (1.2 g/kg) on serum chemistry are shown in Table 3. Ethanol resulted in a transient increase in blood alcohol (detected at 1 and 2 hours), mild hypocalcemia (significant at 4, 8, 12, and 16 hours), and no change in serum immunoreactive parathyroid hormone (*i*PTH).

The maximum induction of mRNA for each of the three bone matrix proteins occurred 6 hours following administration of ethanol. There were transient decreases in mRNA levels for IL-1, IFN-γ, and MIF prior to the increase in mRNA levels for the bone matrix proteins (Figure 6). In contrast, the restoration of normal mRNA levels for the bone matrix proteins corresponded to a significant decrease in the mRNA levels for IGF-I. Ethanol treatment had minimal effects on mRNA levels for pro- and anti-apoptotic genes (Table 4), suggesting that ethanol is not toxic to bone cells.

The transient rise in mRNA levels for bone matrix proteins did not lead to a corresponding increase in bone matrix synthesis. There was no significant difference ($p=0.50$; n=5-6/group) in ^{3}H-proline incorporation into proximal tibial metaphysis between ethanol-treated (297 ± 61 CPM/mg) and solvent-treated (253 ± 28 CPM/mg) rats when the labeled amino acid was administered 6 hours after ethanol treatment (Table 1; Experiment 6).

To determine whether the ethanol-induced transient increases in mRNA levels for bone matrix proteins requires protein synthesis, rats were administered cycloheximide 2 hours prior to ethanol (Table 5). Cycloheximide had no effect on mRNA levels in solvent-treated or ethanol-treated rats. However, the results cannot be easily interpreted because the expected ethanol-induced significant increase in mRNA levels was not observed.

Task 3

The goal of Task 3 is to determine which induces a more detrimental skeletal response: peak blood concentrations of ethanol or chronic elevation of blood alcohol.

We have completed two studies investigating the effects of ip injection and gavage (Table 1). The effects of short-term continuous administration will be completed in Year 2.

The comparative effects of administration of ethanol by daily ip injection (Table 1; Experiment 7) with gavage (Experiment 8) on mRNA levels for bone matrix protein and bone

histomorphometry are shown in Table 6 and Table 7, respectively. Gavage has no effects on steady-state mRNA levels for bone matrix proteins or bone histomorphometry. In contrast, ip injection significantly reduced mRNA levels for Type 1 collagen, osteocalcin and osteonectin, and decreased cancellous bone formation. Similarly, gavage had no effect on mRNA levels for cytokines (Table 6). Ip injection, however, decreased mRNA levels for IGF-1 and IL-6.

Task 4

The goal of this task is to evaluate the long-term skeletal effects of ethanol. We have completed the animal studies (Table 1; Experiment 9) and have performed bone histomorphometry and RNA analysis. The long-term effects of administration of ethanol on serum chemistry, body weight, bone histomorphometry, and gene expression are shown in Tables 8-14.

Pair-feeding has no effect on any measured value. For this reason, the pair-fed and ad lib controls groups were combined.

The dose effects of ethanol on body weight, food and ethanol consumption are shown in Table 8. The initial body weights did not differ between the treatment groups. Ethanol had minor effects on body weight and food consumption. The lowest concentration of ethanol (3% caloric intake) increased final body weight as well as consumption of the diet, whereas the highest concentration (35% of caloric intake) tended to reduce final weight gain ($p<.06$) and significantly decreased consumption. There was a near linear increase in total ethanol consumed/day as the concentration of ethanol was increased in the diet.

The dose effects of ethanol on serum chemistry are shown in Table 9. Ethanol had either no effect or mildly increased total calcium (6% of caloric intake), had no effect on cholesterol and creatinine, and significantly reduced osteocalcin at all but one dose rate (6% of caloric intake).

The dose effects of ethanol on static bone histomorphometry are summarized in Table 10. BV/TV was significantly decreased at 13% and 35% of caloric intake and Tb.Th was decreased at intake levels at and above 6%. Tb.N and Tb.S were not influenced by ethanol. Ethanol treatment resulted in a significant ($p<0.0001$) dose dependent decrease ($r = .31$) in BV/TV as determined by linear regression analysis (data not shown).

The dose effects of ethanol on cancellous bone dynamic and cellular histomorphometry are summarized in Table 11. Ethanol-treatment resulted in progressive dose dependent decreases in MS/BS, BFR/BS and BFR/BS. Ethanol had no effect on MAR or osteoblast perimeter. Ethanol-treatment reduced osteoclast number at all concentrations but the magnitude of the inhibition did not depend upon dose.

The dose effects of ethanol on steady-state mRNA levels for bone matrix proteins are shown in Table 12. The highest concentration of ethanol (35% of caloric intake) uniformly reduced mRNA levels for type 1 collagen, osteonectin and osteocalcin. There were no other significant differences.

The effects of ethanol (6% and 35% of caloric intake) on steady-state mRNA levels for cytokines and growth factors are summarized in Table 13. The mRNAs for TNF-ß, IL-1 receptor antagonist, IL-10, or IFN-ß were not detected in extracts from rat bone from either control or ethanol-treated rats (data not shown). The mRNAs for IL-1α, IL-12, IFN-γ, MIF, TGF-$ß_1$, TGF-$ß_2$, and TNF-α were detected but were not influenced by the treatment. Steady-state mRNA levels for IGF-I and IL-6 were reduced by ethanol treatment.

The long-term effects of ethanol on mRNA levels for pro- and anti-apoptotic genes (Experiment 9) are shown in Table 12. Ethanol resulted in dose dependent decreases in mRNA levels for bcl-x, bax, bcl-2, FAF and RIP. mRNA levels for bcl-x, bax, bcl-2 were decreased in rats fed 6% of their caloric intake as ethanol, whereas, FAF and RIP were decreased at 35%. These results are in agreement with the short-term studies in that they suggest that ethanol is not toxic to bone cells.

Task 5

The goal of Task 5 is to determine the effects of ethanol on skeletal adaptation to resistance exercise training. We have acquired the resistance training equipment and are in the process of validating procedures. We anticipate performing the animal studies in Year 2.

Task 8

The goal of Task 8 is to determine the effects of prior consumption of ethanol on PTH-induced increases in mRNA levels for bone matrix proteins. We have performed one study (Table 1; Experiment 10).

The effects of administration of ethanol (1.2 g/kg) or water 6 hr prior to administration of PTH or vehicle on steady state mRNA levels for bone matrix proteins (Experiment 10) are shown in Table 15. Shown also is the effect of simultaneous administration of ethanol and PTH. Ethanol followed by PTH resulted in a decrease in mRNA levels for Type 1 collagen. Ethanol alone and simultaneous administration of ethanol and water resulted in significant decreases in mRNA levels for osteonectin. Simultaneous administration of ethanol and PTH also reduced mRNA levels for osteocalcin.

(7) KEY RESEARCH ACCOMPLISHMENTS

The detrimental effects of chronic alcohol abuse on the skeleton of humans and laboratory animals are well established (2-10). The present studies demonstrate that even low dose rates of ethanol inhibit bone turnover. A daily caloric intake of ethanol as little as 3% (equivalent to ~1/2 drink/day) significantly inhibited indices of bone turnover. The inhibitory effects of ethanol do not appear to be due to toxicity and thus may be reversible.

The inhibitory effects of ethanol on bone metabolism are associated with transient changes in mRNA levels for bone matrix proteins and cytokines and growth factors. Although acute ethanol treatment results in a transient rise in mRNA levels for bone matrix proteins, this effect is not accompanied by an increase in bone matrix synthesis. Indeed, chronic daily treatment with

ethanol results in decreased mRNA levels for these proteins and reduced bone formation. This inhibition in bone formation is associated with decreased mRNA levels for IGF-I, an important osteoblast growth factor.

(8) REPORTABLE OUTCOMES

We are completing analysis of the findings from Year 1 and anticipate submitting abstracts and manuscripts in Year 2.

(9) CONCLUSIONS

Our Year 1 results are consistent with the initial hypothesis. Indeed, ethanol had effects on bone metabolism at much lower dose rates than anticipated.

We do not anticipate any need for major changes in either the working hypothesis or specific aims of this proposal. The data obtained during Year 1 of the project is consistent with the concept that ethanol has profound effects on bone metabolism. Furthermore, these actions occur at lower dose rates than anticipated. Importantly, the data strongly suggests that ethanol antagonizes osteoblast function but does not appear to be toxic. This finding supports the concept that ethanol interferes with the response of bone cells to extracellular signals such as hormones and mechanical loading. These changes appear to be specific because we do not observe a uniform decrease in expression of cytokines and growth factors. We have identified two putative factors which are consistently altered by ethanol; IGF-I and TNF-α. TNF-α is increased in a dose- and time-dependent manner whereas IGF-I is decreased. These findings are of great interest because TNF-α is positively associated with bone resorption whereas IGF-I is associated with bone formation (11-13). Furthermore, IGF-I is believed to be an important intermediate in PTH as well as mechanical loading induced stimulation of bone formation. Thus, we may have identified a potential molecular mechanism for the detrimental effects of ethanol on bone remodeling.

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(11) APPENDICES

Appendix 1: Tables

Table 1: Summary of Experiments Performed		
Experiment #	Title	Task Number(s)
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9	Long-term dose response effects of ethanol on bone histomorphometry and gene expression	4
10	Effect of prior consumption of ethanol on PTH-induced increases in mRNA levels for bone matrix proteins	8

Table 2: Dose Response Effects of Ethanol on Blood Alcohol		
Dose (g/kg)	Serum Ethanol (mg/dL)	P value vs. control
Control	ND	
0.15	ND	NS
0.3	ND	NS
0.6	20±3	< .01
1.2	94±2	< .001
1.7	170±15	< .001
Values are mean ± SE; n=4-5/group. ND is below detection limit. Blood alcohol levels were determined 1 hr following ethanol administration. Blood alcohol was below the detection limit at all doses when determined 6 hr following ethanol administration.		

Table 3.-- The Time Course for Effects of Ethanol (1.2 g/kg) on Serum Chemistry			
Time (hr)	Ethanol (mg/dl)	Calcium (mg/dl)	*i*PTH (ng/ml)
0	--	11.6±.2	198±44
1	94±2*	11.0±.1	175±65
2	55±14*	11.1±.2	115±20
4	--	10.7±.2*	167±39
6	--	11.3±.1	214±74
8	--	10.9±.2*	288±30
12	--	10.2±.3*	210±43
16	--	10.6±.2*	196±29
Values are mean ± SE; n=5-6, except 12 hr timepoint where n=10. *p<.05 vs 0 time. -- indicates values below the detection limit.			

Table 4: Time Course Effects of Ethanol on Expression of Pro- and Anti-Apoptotic Genes						
Gene	bcl-w	bcl-x	bak	bax	bcl-2	bad
Control	.007±.001	.050±.005	.040±.004	.278±.024	.186±.011	.064±.003
2 hr	.009±.001*	.052±.006	.038±.004	.280±.021	.193±.016	.057±.002
4 hr	.012±.001*	.052±.006	.038±.003	.321±.017	.213±.009	.063±.003
6 hr	.009±.001	.055±.009	.044±.005	.261±.054	.203±.014	.059±.003
8 hr	.011±.001*	.221±.163	.045±.004	.426±.106	.279±.046	.073±.011
12 hr	.009±.001	.048±.007	.037±.005	.259±.015	.193±.008	.056±.002
16 hr	.009±.001*	.041±.005	.031±.003	.295±.025	.193±.001	.068±.012
24 hr	.004±.001	.038±.005	.028±.003	.347±.041	.215±.018	.055±.006
Values are mean ± SE; n=4. *$p<0.05$ compared to control.						

Table 5: Lack of an Acute Effect of Ethanol and/or Cycloheximide on mRNA Levels for Bone Matrix Proteins			
Gene	Type 1 Collagen	Osteocalcin	Osteonectin
Control	.98±.13	1.16±.47	1.11±.21
Ethanol	.72±.11	1.70±.54	1.30±.31
CHX+Ethanol	1.12±.20	1.49±.48	1.34±.35
CHX	1.34±.22	1.29±.38	1.28±.30
Values are means ± SE; n=6. CHX is cycloheximide.			

Table 6: Comparative Effects of Ethanol Administered by ip Injection with Gavage on mRNA Levels for Bone Matrix Proteins, Cytokines and Growth Factors		
mRNA	i.p.	Gavage
Bone Matrix Proteins		
Osteonectin	↓ ($p < .002$)	NC
Osteocalcin	↓ ($p < .0001$)	NC
Type 1 Collagen	↓ ($p < .0002$)	NC
Cytokines and Growth Factors		
IGF-I	↓ ($p < .015$)	NC
TNF-α	NC	NC
TGF-β_1	NC	NC
TGF-β_2	NC	NC
IL-12	NC	NC
IL-1ß	NC	NC
IL-Ra	NC	NC
IFN-γ	NC	NC
MIF	NC	NC
IL-6	↓ ($p < .03$)	NC
Arrow points in direction of change. NC = no change.		

Table 7: Comparative Effects of Daily Ethanol Administration for 1 Week by ip Injection with Gavage on Bone Histomorphometry		
Measurement	i.p.	Gavage
Cortical Bone		
Cross-sectional area	NC	NC
Medullary area	NC	NC
Cortical area	↓ ($p < .07$)	NC
Periosteal BFR	↓ ($p < .0003$)	NC
Periosteal MAR	↓ ($p < .001$)	NC
Periosteal MS	↓ ($p < .0002$)	NC
Cancellous Bone		
BV/TV	NC	NC
MS/BS	NC	NC
MAR	↓ ($p < .025$)	NC
BFR/BS	↓ ($p < .0025$)	NC
Cortical measurements were performed at the tibia fibula junction. Cancellous bone measurements were performed in the secondary spongiosa of the proximal tibia metaphysis. Bone formation rate (BFR), mineral apposition rate (MAR), mineralizing surface (MS), bone volume (BV), tissue volume (TV), arrow points toward direction of change. NC = no change.		

Table 8: Body Weights and Diet Consumed				
Group	Initial BW (g)	Necropsy BW (g)	Diet consumed (ml/d)	Ethanol consumed (ml/d)
Control	277±5	321±9	67.8±2.3	0
3% Ethanol	279±6	358±15*	76.6±1.5*	0.44±0.01*
6% Ethanol	281±5	339±12	69.3±1.4	0.80±0.02*
13% Ethanol	272±9	323±15	64.1±1.3	1.60±0.03*
35% Ethanol	285±6	291±7**	61.5±2.4*	4.12±0.16*

Table 9: Serum Measurements				
Group	Total Calcium (mg/dl)	Cholesterol (mg/dl)	Osteocalcin (ng/ml)	Creatinine (mg/dl)
Control	10.9±0.1	139.8±3.4	78.5±3.8	0.782±0.020
3% Ethanol	11.1±0.1	134.9±6.1	62.6±5.0*	0.793±0.028
6% Ethanol	11.3±0.1*	149.2±7.5	80.5±3.7	0.823±0.027
13% Ethanol	11.0±0.1	151.1±8.5	67.0±3.9*	0.825±0.033
35% Ethanol	10.9±0.1	153.5±9.6	59.8±3.5*	0.855±0.025
Values are mean ± SEM (n=9-12). * $p<0.05$ vs. control (n=19).				

Table 10: Cancellous Bone Static Histomorphometry				
Group	BV/TV (%)	Tb.Th (μm)	Tb.N (mm^{-1})	Tb.S (μm)
Baseline	25.6±1.3	70.1±1.9	3.7±0.2	278±13
Control	20.6±1.2	64.9±2.6	3.2±0.1	325±14
3% Ethanol	19.5±1.4	62.5±3.0	3.1±0.1	330±15
6% Ethanol	18.3±1.7	57.6±2.3*	3.2±0.2	332±26
13% Ethanol	15.7±2.3*	52.5±2.4*	2.9±0.4	411±71
35% Ethanol	15.8±1.0*	50.9±1.7*	3.1±0.1	332±19
Values are mean ± SEM. * $p < 0.05$ treatment (n=8-11) vs. control (n=19). Bone volume (BV)/tissue volume (TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.S).				

Table 11: Cancellous Bone Dynamic and Cellular Histomorphometry						
Group	MS/BS (%)	MAR (μm/d)	BFR/BV (%/d)	BFR/BS (%/d)	Ob.S/BS	Oc.S/BS
Control (n=20)	11.05±0.75	0.94±0.04	0.325±0.019	0.104±0.008	5.61±1.03	15.5±1.2
3% Ethanol	5.79±1.07*	0.92±0.02	0.169±0.032*	0.054±0.011*	2.85±0.65	10.8±1.8*
6% Ethanol	5.14±0.78*	0.91±0.03	0.171±0.031*	0.047±0.008*	4.61±0.81	10.6±1.2*
13% Ethanol	3.71±0.77*	1.01±0.04	0.150±0.035*	0.037±0.008*	4.79±0.94	10.7±1.0*
35% Ethanol	2.37±0.56*	0.86±0.04	0.077±0.019*	0.020±0.006*	2.97±0.52	10.1±1.5*
Values are mean ± SEM (n=8-11); * p<0.05 vs. control (n=19-20). Mineralizing surface (MS), bone surface (BS), mineral apposition rate (MAR), bone formation rate (BFR), bone volume (BV).						

Table 12: The Long Term (4 months) Dose Response Effects of Ethanol on Steady-State mRNA Levels for Bone Matrix Proteins			
Gene/Dose Rate	Type 1 Collagen	Osteonectin	Osteocalcin
Control	2.69±.04	1.37±.07	.192±.026
3% Caloric Intake	3.11±0.	.171±.022	.204±.029
6% Caloric Intake	.272±.120	.132±.027	.159±.039
13% Caloric Intake	.152±.038	.105±.020	.175±.022
35% Caloric Intake	.062±.020*	.051±.007*	.098±.014*
Values are mean ± SE; n = 10. *p < 0.05 compared to control.			

Table 13: Effect of Ethanol (35% caloric intake) on steady-state mRNA Levels for Cytokines and Growth Factors in the Proximal Tibial Metaphysis.

Cytokine/Growth Factor	Change
IL-1α	NC
IL-6	↓*
IL-12	NC
IGF-1	↓*
IFN-γ	NC
MIF	NC
TGF-B_1	NC
TGF-$ß_2$	NC
TNF-α	NC

* $p < .05$ compared to controls.

Values were normalized to L32; (N=3).

No significant change (NC), decreased (↓), interleukin (IL), insulin-like growth factor (IGF), interferon (IFN), macrophage inhibitory factor (MIF), transforming growth factor (TGF), tumor necrosis factor (TNF).

Table 14: Dose Response Effects of Long-Term (4 months) Ethanol Treatment on mRNA Levels for Pro- and Anti-apoptotic Genes		
Gene	6% Caloric Intake	35% Caloric Intake
bcl-w	NC	NC
bcl-x	↓ ($p < .025$)	NC
bak	NC	NC
bax	↓ ($p < .002$)	NC
Bcl-2	NC	NC
Bad	NC	NC
FAP	NC	↓ ($p < .015$)
RIP	NC	↓ ($p < .05$)
Arrow points in direction of change. NC = no change.		

Table 15: The Effects of Prior and Simultaneous Ethanol Treatment on mRNA Levels for Bone Matrix Proteins in PTH-Treated Rats			
Gene	Type 1 Collagen	Osteonectin	Osteocalcin
Control	4.3±0.7	0.94±0.08	0.90±0.10
Ethanol followed by PTH	2.4±0.2*	0.73±0.09	0.59±0.09
Ethanol	2.8±0.4	0.66±0.07*	0.79±0.19
PTH	3.1±0.2	0.78±0.06	0.64±0.06
Ethanol and PTH	2.5±0.6	0.51±0.10*	0.51±0.10*
Values are mean ± SE; n = 4 *$p < 0.05$ compared to control			

APPENDIX 2: Figures

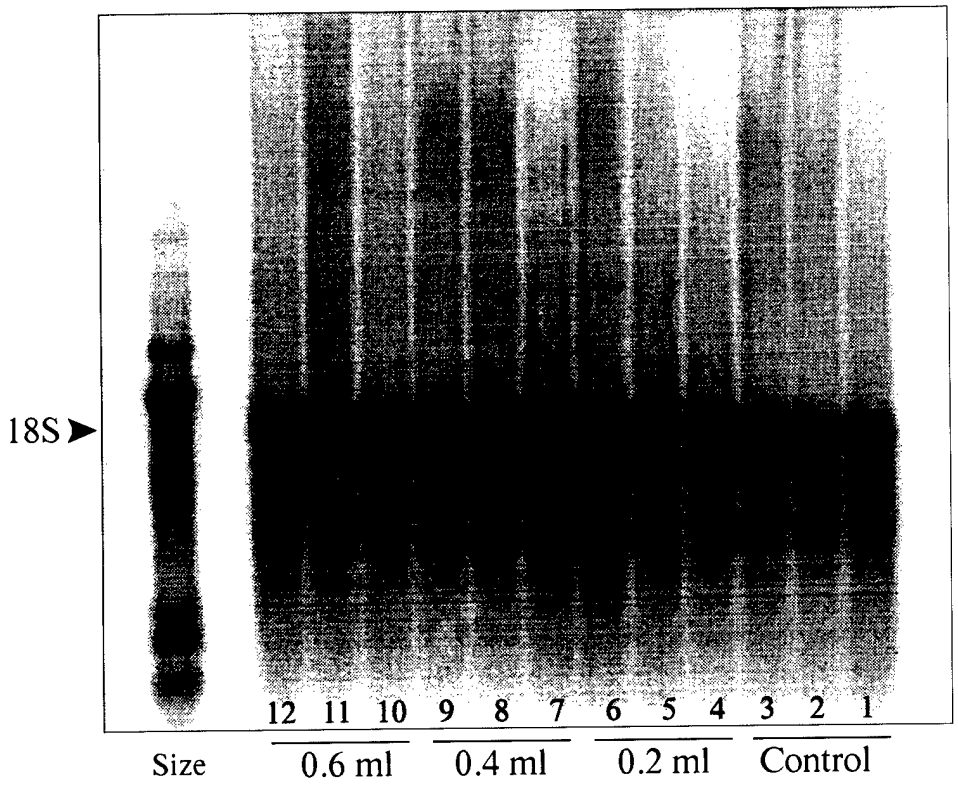


Figure 1

A. Representative Northern analyses for 18S ribosomal RNA. Ten μg of total cellular RNA was added to each lane and separated electrophoretically prior to transfer to a membrane and hybridization with a cDNA probe for 18S. The RNA in each lane was from a different animal. The results contribute to the data showing no effect of increasing doses of alcohol on 18S.

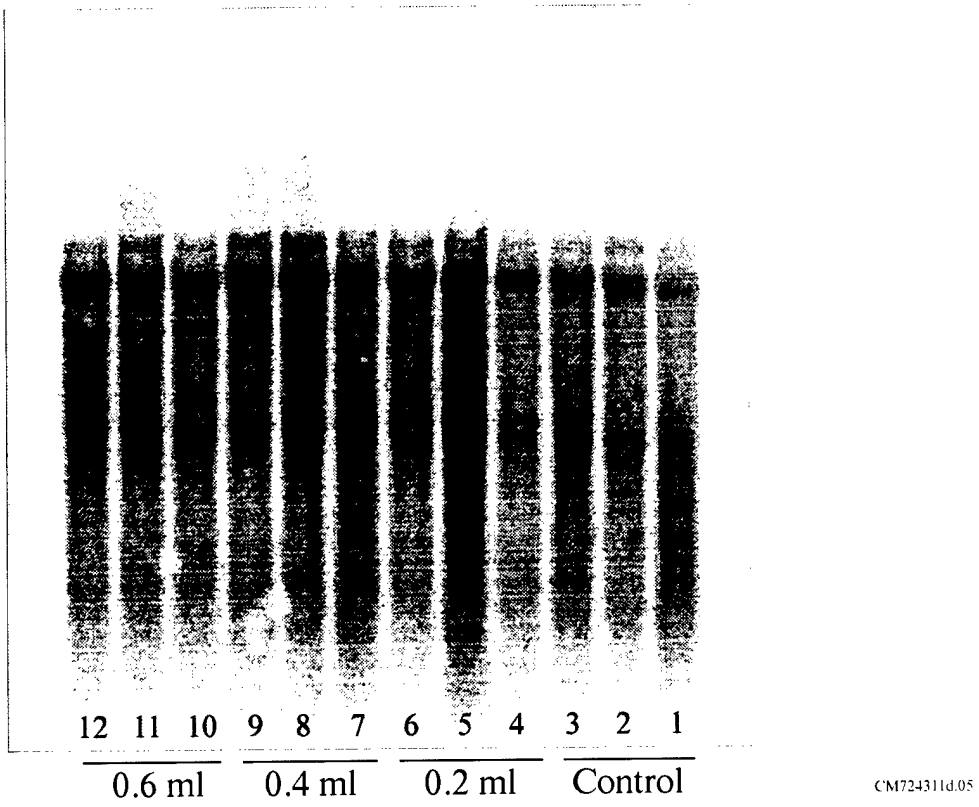


Figure 1

B. Northern analyses for collagen. The same membrane as in A was hybridized with a cDNA probe for collagen. The quantitative results from multiple similar assays are summarized in Figures 2-4.

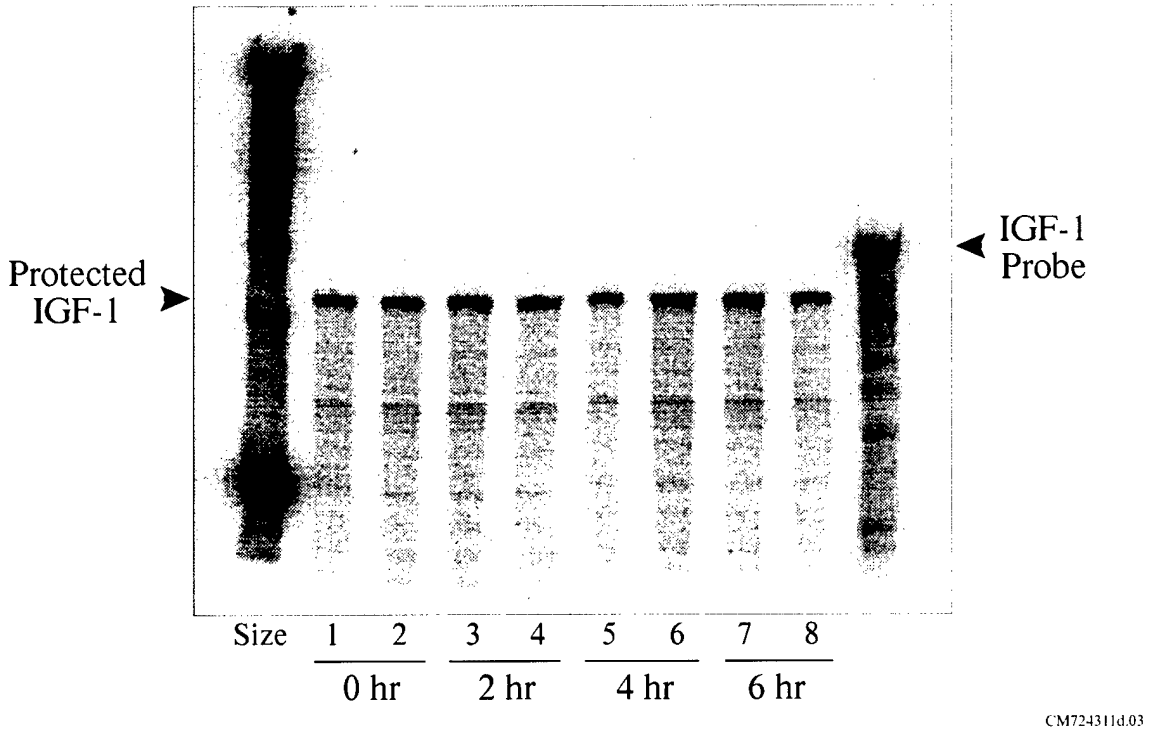


Figure 1

C. RNase protection assay for IGF-1. Ten µg of total cellular RNA was hybridized with a riboprobe for IGF-1 and treated with RNase prior to electrophoresis. Size refers to size markers. The last lane to the right shows the size of the undigested probe. The results from multiple quantitative similar assays are summarized in Figs. 5 and 6.

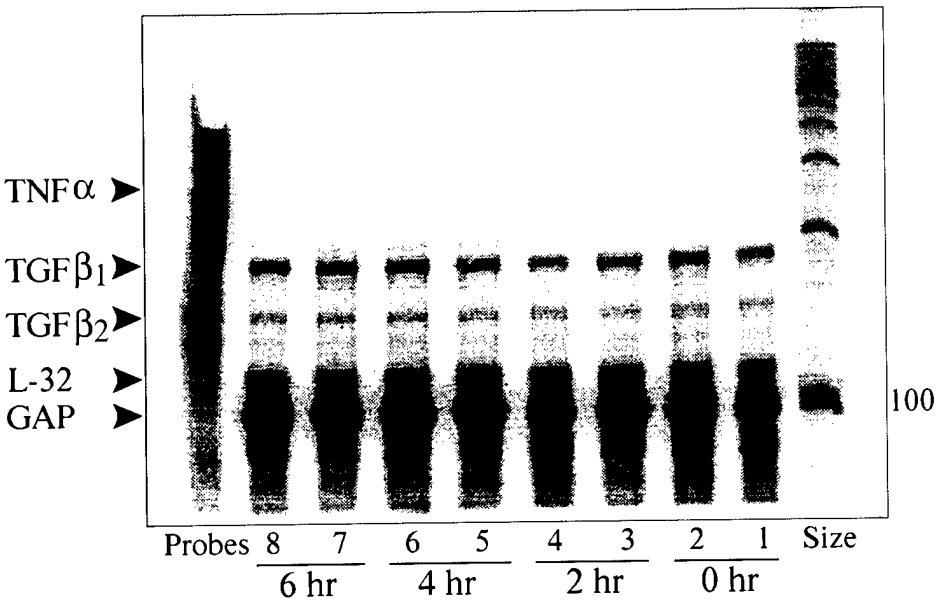
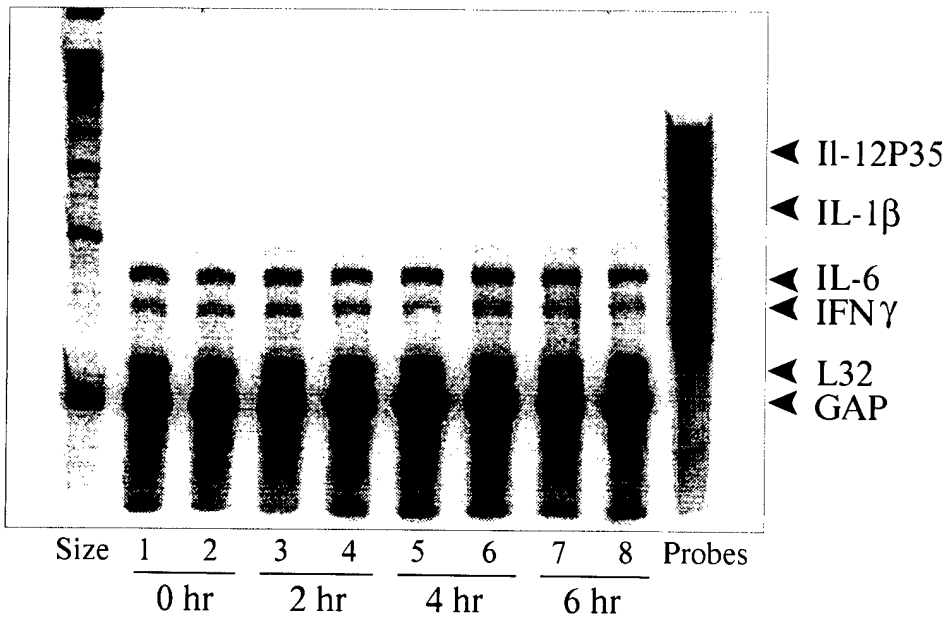


Figure 1
D. RNase protection assay showing GAP, L32, TGF-$ß_1$, TGF-$ß_2$ and TNF-α. The quantitative results of multiple similar assays are summarized in Figs. 5 and 6.



CM724311d.04

Figure 1
E. RNase protection assays showing GAP, L32, IFN-γ, IL-6, IL-ß, and IL-12 P35. The quantitative results of multiple similar assays are shown in Figs. 5 and 6.

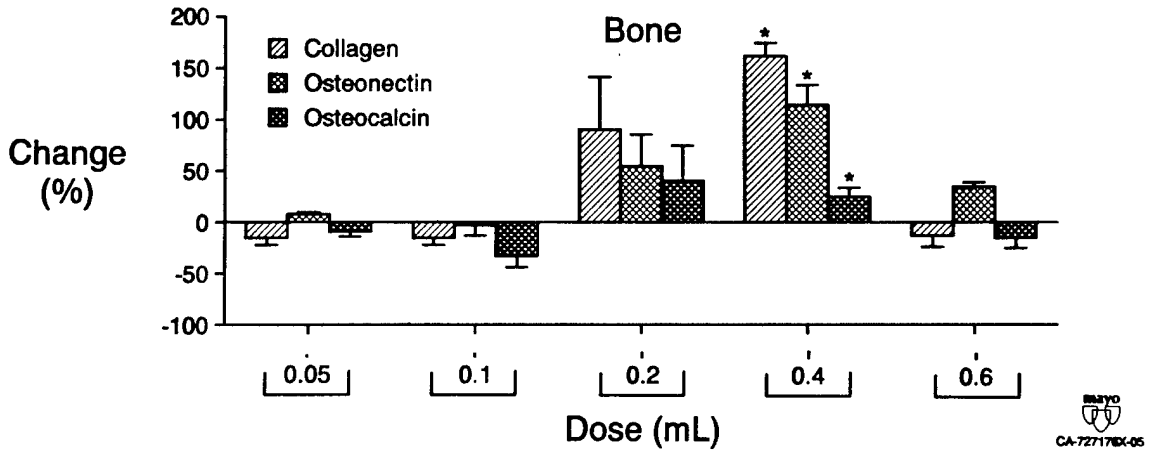


Figure 2.--The dose response effects of ethanol on steady-state mRNA levels for matrix proteins in bone. The tissues were retrieved 6 hr following administration of 0.05-0.6 ml (0.15-1.7 g/kg) of ethanol. Values are mean ± SE; N= 4-5 per group. ND is not detected. * $p < 0.005$ vs. 0 dose.

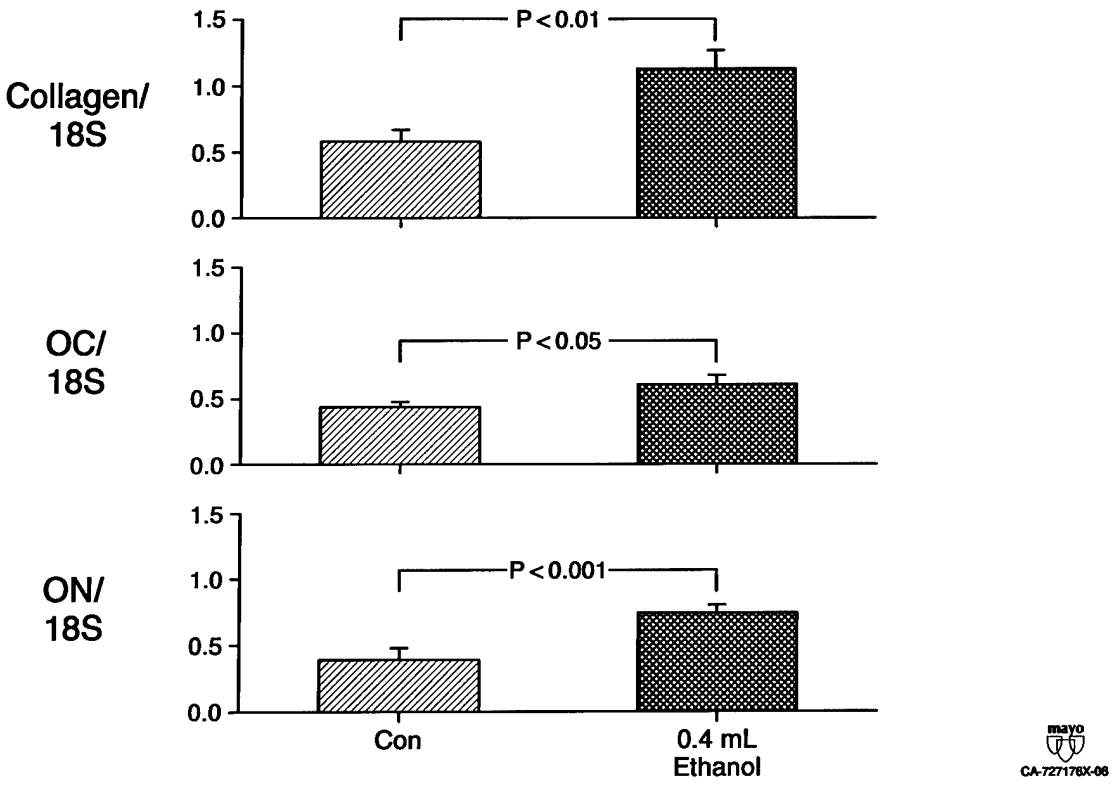


Figure 3.--Ethanol increases steady-state mRNA levels for matrix proteins in vertebrae 6 hr after intraperitoneal administration of 0.4 ml of alcohol. Values are mean ± SE; N=4-5. OC, osteocalcin; ON, osteonectin; Con, control.

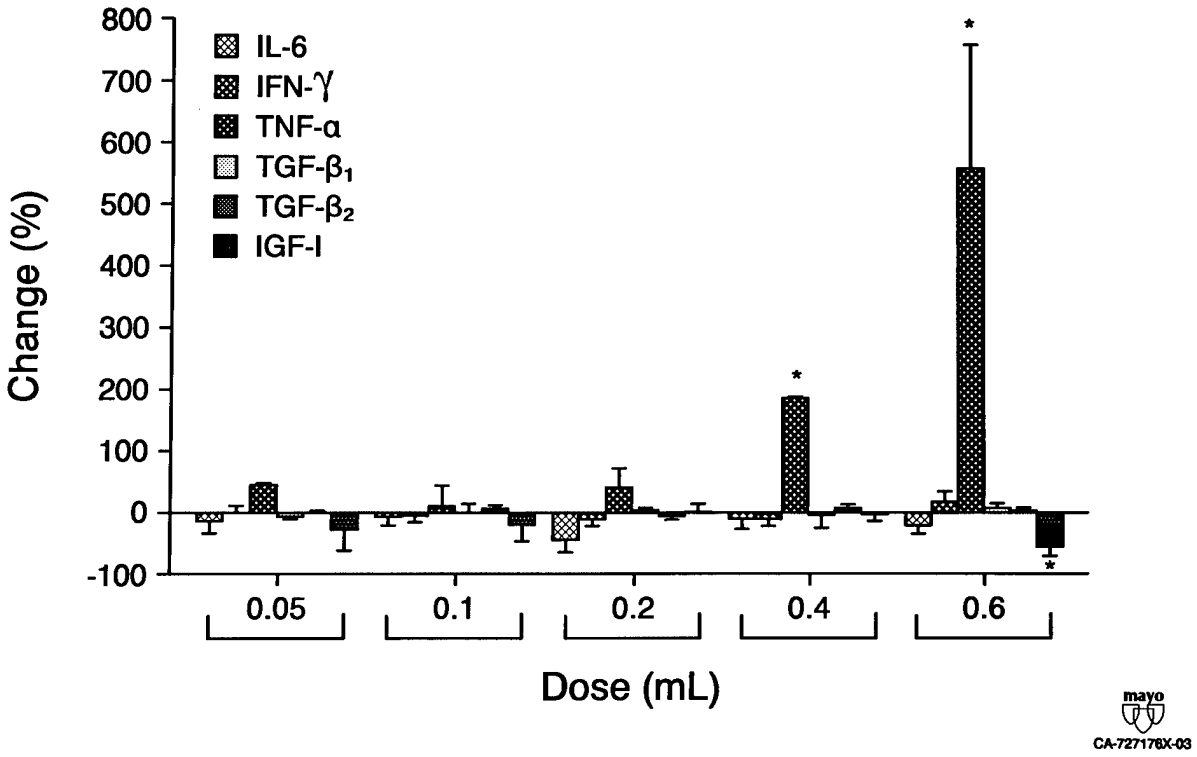


Figure 4.—Dose-response effects of ethanol on steady-state mRNA levels for growth factors and cytokines in bone. Tissues were retrieved 6 hr after administration of 0.05 to 0.6 ml (0.15 to 1.7 g/kg) of ethanol. Values are mean ± SE; N= 4-5/group. *$p < 0.05$ vs. 0 dose.

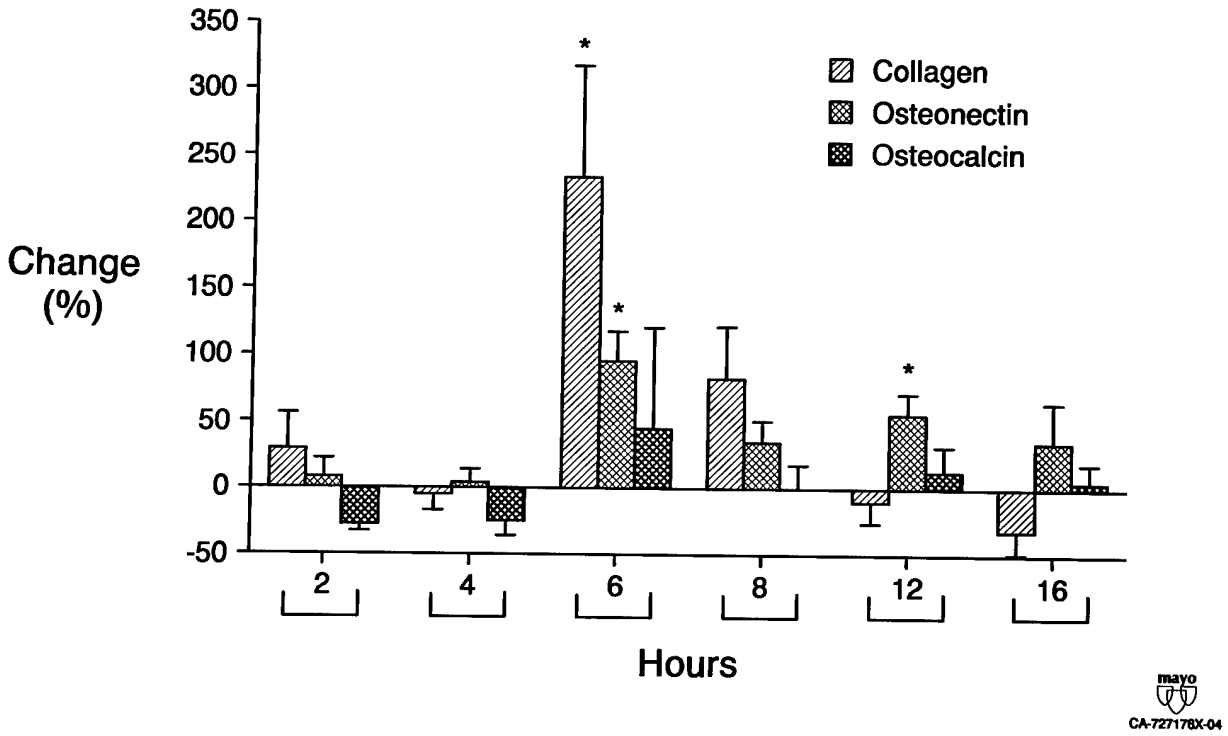
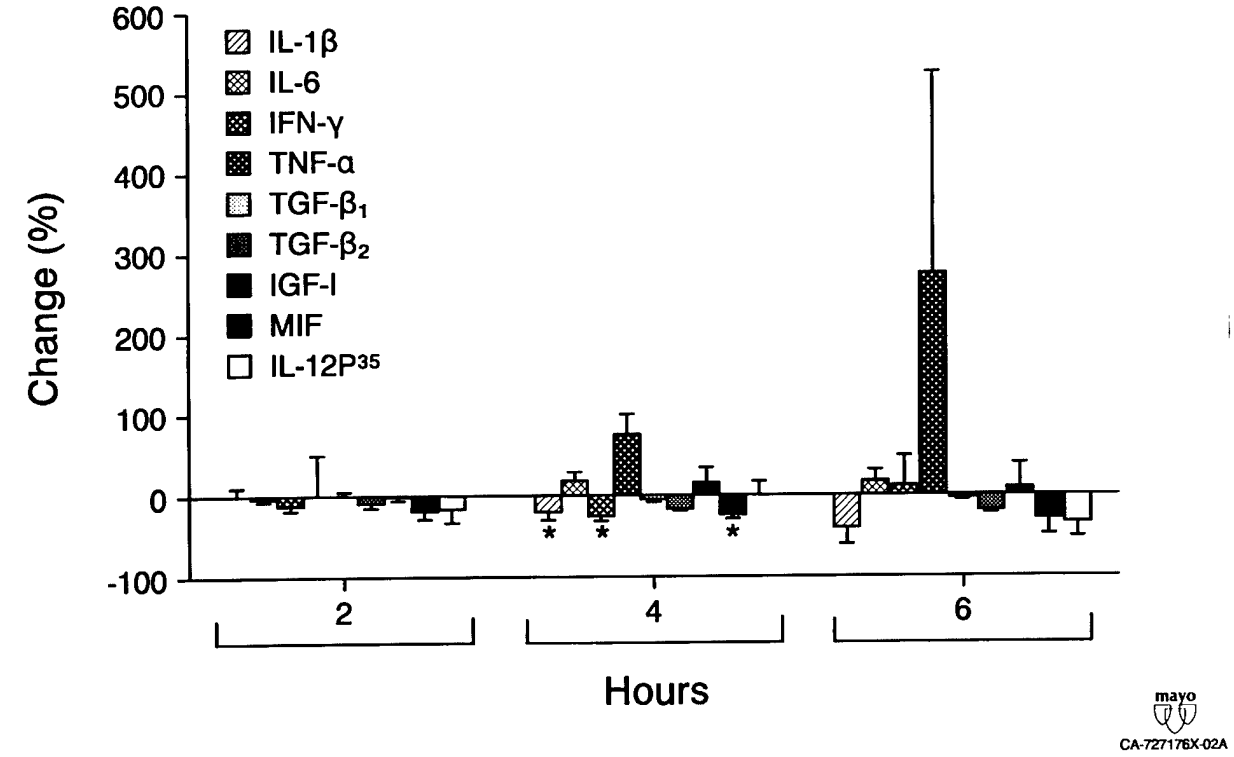


Figure 5.--The time course effects of ip administration of 1.2 g/kg of ethanol on steady-state mRNA levels for matrix proteins in bone. Values are mean ± SE; N= 4-5 per group. *$p < 0.005$ vs. 0 time.



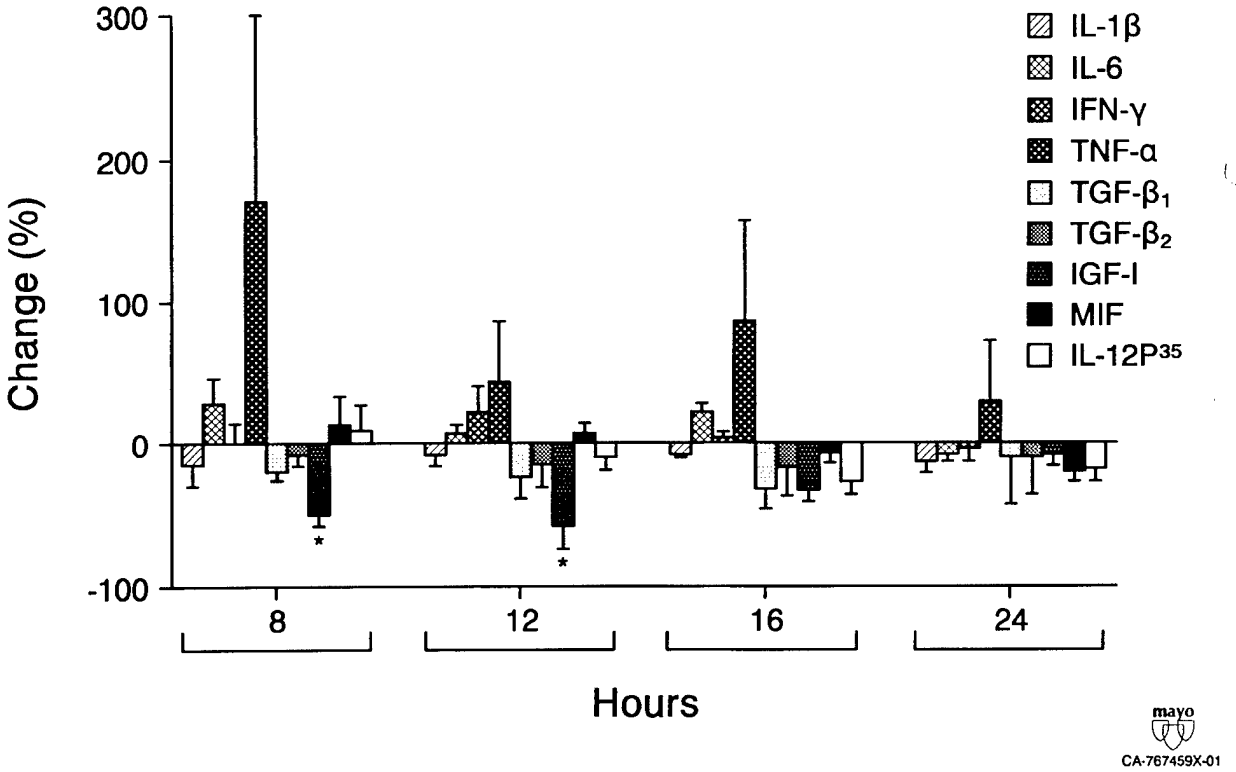


Figure 6.--The time course effects of ethanol on steady-state mRNA levels for cytokines and growth factors in proximal tibial metaphysis. (A) 2-6 hr. (B) 8-24 hr. Values are mean ± SE; N= 4-5/group. *$p < 0.05$ vs. 0 time.